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(71) Applicant: UNIVERSITE DE MONTREAL [CA/CA]; 2900 Edouard Montpetit, Montréal, Québec H3T 1J4 (CA).

(72) Inventors: ELAZHARY, Youssef; 1965 de Carrillon, St-Hyacinthe, Québec J2S 7G6 (CA). CORNAGLIA, Estela; 2155 Grand Rang, St-Charles, Québec J0H 2G0 (CA). CHARARA, Souhel; 555 Octave Crémazie #6, St-Hilaire, Québec J3H 5V5 (CA).

(74) Agent: COTE, France; Swabey Ogilvy Renault, Suite 1600, 1981 McGill College Avenue, Montréal, Québec H3A 2Y3 (CA).

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(54) Title: DIAGNOSTIC KIT FOR BOVINE SYNCYTIAL RESPIRATORY VIRUS

(57) Abstract

The present invention relates to an ELISA test for qualitatively determining the presence of living or inactivated BRSV antigen in a bovine biological sample, wherein the sample has an unknown amount of BRSV antigen, which comprises the following: a) incubating a solid support having bound thereto a first anti-BRSV antibody with the biological sample for a time sufficient for an immune complex to form between the anti-BRSV antibody and any BRSV antigen present in the sample; b) incubating the incubated solid support of step a) with a second anti-BRSV antibody; and c) detecting the bound second antibody of step b) to determine the quantity of the BRSV antigen present in the sample.

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DIAGNOSTIC KIT FOR BOVINE SYNCYTIAL RESPIRATORY VIRUS

BACKGROUND OF THE INVENTION

(a) Field of the Invention

The invention relates to an ELISA test which permits a specific, sensitive and rapid diagnostic of the presence bovine respiratory syncytial virus.

(b) Description of Prior Art

The bovine respiratory syncytial virus (BRSV) was isolated in 1970. This virus belongs to the genes Pneumovirus and to the family *Paramyxoviridae*. It is encapsulated, pleomorphic virus which is composed of a single branch ribonucleic acid (ARN). The BRSV is made of 10 proteins in which two are important for viral replication (Huang, Y. et al., 1985, *Virus Res.*, **2**: 157-173). These are glycoprotein F which is responsible for fusion and protein G which is responsible for viral attachment (Walsh, E. and Hiruska, J., 1983, *J. Virol.*, **47**:171-176). These two proteins as well as protein M and a polypeptide 22Kd are in the envelop. Nucleocapside is made of ARN and three proteins: nucleoprotein (N), phosphoprotein (P) and polymerase (L). The main antigenic differences, among the strains of bovine and human origin, as well between the human strains belonging to group A or B, are found on the protein G (Baker, J. et al., 1992, *J. Clin. Microbiol.*, **30**: 1120-1126; Garcia-Barreno, B. et al., 1989, *J. Virol.*, **63**: 925-932; Lerch, R. et al., 1989, *J. Virol.*, **63**: 833-840). On different strains, protein N and protein F have shown a high degree of homology with respect to their sequence of amino acids (Amann, V, et al., 1992, *J. Gen. Virol.* **73**: 999-1003; Garcia-Barreno, B. et al., 1989; Lerch, R. et al., 1989, *J. Virol.*, **63**: 833-840; Mulkey, K. and Anderson, G., 1991, *J. Clin. Microbiol.*, **29**: 2038-2040).

The infection caused by BRSV has been described as the most important sickness causing an acute inflammation of the respiratory tracts in dairy cows and calves which are less than 12 months old (Elazhary et al., 1982, *Cornell Vet.*, **72**: 325-333). The first symptoms of the sickness appear in the form of cough, pyrexia and a nasal and ocular exsudat. BRSV may cause anorexia, trachypnea and dyspnea which may lead to terminal pneumonia. An infection by BRSV, which is extremely contagious, spread horizontally by direct contact of rejects of respiratory tracts of infected animals. Morbidity is very high. The infected animals suffer a lost of weight as well as a decrease of milk production and they are more susceptible to secondary infections (Elazhary, Y. et al., 1982, *Cornell Vet.*, **72**: 325-333). The economical losses caused by morbidity and the death rate of this infection are therefore very high, in particular in intensive breeding. In Canada, these losses are estimated at more than 50 million dollars per year.

An efficient control of calves require the fast and reliable detection of a sickness in order to enable the isolation of affected animals and to decrease the risk of spreading of the disease to none infected flocks.

The tracking down of the infection in bovine may be carried out by viral isolation or by the detection of the virus in the nasal epithelial cells which are colored by the technique of immunofluorescence. These two techniques require the availability of a laboratory and qualified personnel. Moreover, because of the very fragile nature of the BRSV virus, the viral isolation is applicable only in the case where the inoculation may be carried out immediately after collecting the sample. It has been established that the termination

of the presence of the antigen in the nasal epithelial cells is at least as sensitive as the isolation and consequently this technique has been accepted as current practice in laboratories.

It would be highly desirable to be provided with a test for use in the field and in the laboratory and may rapidly determine the presence of the living or inactivated BRSV.

It would be highly desirable to be provided with an ELISA test which permits a specific, sensitive and rapid diagnostic of the presence BRSV.

SUMMARY OF THE INVENTION

One aim of the present invention is to provide a test for use in the field and in the laboratory and may rapidly determine the presence of the living or inactivated BRSV.

Another aim of the present invention is to provide an ELISA test which permits a specific, sensitive and rapid diagnostic of the presence BRSV.

In accordance with one embodiment of the present invention, the BRSV ELISA test essentially comprises the following:

a) A polyclonal antibody which is specific to the BRSV is adsorbed on the surface of the wells of the ELISA plate.

b) After rinsing and removing the unbound antibody, a blocking solution is used to obstruct the non-covered sites thus preventing adhesion of any other particle at the surface of the wells. To eliminate the excess of the blocking solution and to set the exposure of the polyclonal antibody to the antigen particles, another washing is carried out.

c) A sample is incubated on the ELISA plate where only the antigen particles will bind to the antibody to

which they are specific. The number of viral particles bound to the polyclonal antibody is dependent on the concentration of the virus in the sample.

d) Washing will permit the removal of the sample and of the particles which are not bound.

e) A second antibody, which, this time is a monoclonal antibody (MAb) is placed in the presence of the virus. Since this antibody is specific to the antigen, it will bind only to the virus which is held on the polyclonal antibody. Another washing will remove any excess of the MAb.

f) Finally, an anti-mouse antibody conjugated to peroxidase will be fixed on the bound MAb. A last washing will remove the excess of unbound anti-mouse antibody. Presence of peroxidase will thereafter enable the development of a blue coloration when the chromogenic substrates are added. The intensity of the coloring will be depend on the quantity of virus present in the sample. Presence of a coloring will be detected visually in the field and by spectrophotometer in the laboratory.

The BRSV ELISA test of the present invention relies on an indirect immuno-enzymatic technique for the detection of an antigen (structural nucleoprotein) which is common to all the strains of the syncytial respiratory virus in the bovine (BRSV). This test, directly carried out on a sample of nasal secretions, enables the determination of the virus BRSV.

The BRSV ELISA test of the present invention essentially includes 4 steps:

1. The controls and the samples to be tested are distributed in cupolas sensitized with anti-BRSV polyclonal antibodies. The viral protein, if present in the sample, is fixed on the specific sites.

2. After a series of washings to eliminate the free or unbounded material, a MAb anti-BRSV antibody (structural nucleoprotein) is added. It is fixed on the antigen which is previously fixed to the polyclonal antibody.

3. After another series of washings, mice IgG of anti-IgG goat coupled to a peroxidase enzyme enable to reveal the binding of the MAb by forming the following complex:

Polyclonal Antibody - Ag RSV (nucleoprotein) - MAb - Conjugate (peroxidase)

4. After the last washings, the enzyme bound to the complex is revealed by addition of a chromogenic substrate which it converts into a blue colored product. A blue color indicates the presence of antigen in the sample. The intensity of the blue color is directly in relation to the quantity of viral antigen present in the sample. In the field, a visual reading of this coloration is carried out. In the laboratory, the reading is carried out by a spectrophotometer after having added a solution to stop the reaction of the substrate.

In accordance with the present invention, there is provided an ELISA test for qualitatively determining the presence of living or inactivated BRSV antigen in a bovine biological sample, wherein the sample has an unknown amount of BRSV antigen, which comprises the following:

a) incubating a solid support having bound thereto a first anti-BRSV antibody with the biological sample for a time sufficient for an immune complex to form between the anti-BRSV antibody and any BRSV antigen present in the sample;

b) incubating the incubated solid support of step a) with a second anti-BRSV antibody; and

c) detecting the bound second antibody of step b) to determine the quantity of the BRSV antigen present in the sample.

The ELISA of the present invention may further comprise a step consisting of washing between steps a) and b). Also, the ELISA of the present invention may further comprise an additional step after the washing and before step b), wherein the step consists of reacting the washed solid support of step a) with a blocking solution to obstruct sites the non-covered by the first antibody, thereby preventing adhesion of any other particle on the solid support.

The preferred blocking solution, which may be used in accordance with the ELISA of the present invention, is selected from the group consisting of TRIS-casein-TWEEN™ and PBS-TWEEN™-thimerosal.

The ELISA of the present invention may further comprise a step consisting of washing before step b), which is preferably effected between steps b) and c).

The preferred first antibody used in accordance with the ELISA of the present invention is a polyclonal antibody, while the preferred second antibody used is a monoclonal antibody.

The detecting step carried out in step c) in accordance with the ELISA of the present invention is effected by fixing an anti-mouse antibody conjugated to peroxidase on the bound antibody. Also, the ELISA may further comprise a a step consisting of washing after fixing the conjugated peroxidase. The conjugated peroxidase will develop a blue coloration upon addition of the chromogenic substrates. The intensity of the bleu color is read visually or by a spectrophotometer after addition of a stopping solution. The preferred stopping solution is H_2SO_4.

The preferred bovine biological sample, which may be used in accordance with the ELISA of the present invention, consists in nasal secretions.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 illustrates the protein specificity of the MAb RSQ89C;

Fig. 2 illustrates the evaluation of the binding of IgG bovines specific to BRSV on the wall of the wells; and

Fig. 3 illustrates the determination of the immunological specificity of the ELISA Test.

DETAILED DESCRIPTION OF THE INVENTION

The first object of the present invention was the production of reagent required for the development of the ELISA test. For this purpose, polyclonal and monoclonal antibodies (MAb) specific to BRSV have been produced and characterized.

The second object of the present invention was the standardization of the ELISA test. The optimum dilution of the polyclonal and MAb was established. The blocking solution, solutions for diluting each reagent and times of incubation have been determined.

The third objective was based on the evaluation of the ELISA test. The intra and inter-plate variation, the sensitivity and immunological specificity as well as the relative sensitivity and specificity have been studied.

The fourth object of the present invention was to establish the conditions and times of preservation of the kit. For this purpose, different solutions and temperatures of preservation as well as the use or non-use of lyophilization have been tested.

I- PRODUCTION OF REAGENTS

1. Production of polyclonal antibodies

1.1. Immunization of the animals

The animals used for the production of polyclonal antibodies were hosting cows between 3 and 6 years old and 300 to 500 kg which show no clinical signs.

The antibodies in the sera from the cows were tested by immunofluorescence technique against the following viruses as antigen: syncytial respiratory virus, bovine viral diarrhea virus, parainfluenza virus, and bovine adenovirus. The cows which were found to be serologically negative to the above-mentioned antibodies have been used. The immunization of seronegative cows was carried out with the BRSV Q1 strain at the concentration of 10^6 $DICT_{50}$/ml.

Each bovine was inoculated with 5 ml, at three different locations, by intramuscular injections. The injections were repeated twice at two weeks intervals. The titer of the antibody against RSV was determined by the ELISA test described below in section I-1.2.

1.2 ELISA technique for testing the bovine polyclonal antibody

Purification of BRSV

The viral suspension, obtained as described in section I-1.1, was clarified by centrifugation at 10 000 g, during 15 minutes. The supernatant was collected while taking note of the volume. Thereafter, four volumes (V) of viral supernatant were mixed with 1V of polyethylene glycol (PEG) 50% W/V in the buffer TEN (TRIS HCl 0.05M, EDTA 0.001M, NaCl 0.15M, pH 7.5) and the mixture was incubated at 4°C, while stirring, during 2 hours and 30 minutes.

Then, centrifugation was carried out at 10 000g, during 30 minutes at 4°C. The supernatant was eliminated and the pellet was diluted in buffer TEN of pH

7.5 at 1/50 of the initial volume of the viral supernatant.

This new mixture was concentrated by ultracentrifugation at 100 000g, on a sucrose cushion 30% and 60% W/W diluted in buffer TEN during 2h, in a centrifuge Beckman L8-55R™ (Beckman Instrument, California, USA), by means of a SW28™ rotor. The viral band was collected and dialyzed in a buffer TRIS 10mM at pH 7.2, at 4°C, while steering, during 12 to 18 hours. The purified virus obtained is titrated, divided into small amounts and frozen at -70°C.

ELISA test

A viral solution of RSV was prepared containing 2 µg of viral protein per 100µl, in a carbonated buffer (Chart No. 1).

Nunc Polysorp F16™ plates having 96 wells were used (Gibco Laboratories, Ontario, Canada # 467679 Lot 131201). In each well 100µl/wells of the viral dilution was deposited. The plates were incubated, during 1 hour at 37°C. The plates were washed three times, then they were frozen at -70°C, wrapped in aluminum foil until ready for use. When needed, they are washed twice with the PBS and dried by inversion.

Bovine serum was diluted 1/100 to 1/218700 (dilution factor of 3 and 8 dilutions per sample) in PBS. 100µl of each dilution were deposited in two wells and they were incubated, during 30 minutes at 37°C. Three washings then followed, with the washing solution (Chart No. 1) and drying was carried out by inversion. 100µl of anti-bovine rabbit antibodies IgG conjugated to peroxidase were added in each well, (Cappel-Oragon Tecknika, Ontario, Canada #3202-0082, lot 33703). After an incubation of 2 hours at 37°C, the product was washed three times. 100µl of substrate-chromogen were added in each well, reaction was

allowed to take place during 10 minutes, and the reaction was stopped with 30µl of H_2SO_4 (4N).

The plate was read by bichromatism, at a wave length of 450nm with a reference of 550nm, on a spectrophotometer for ELISA (SLT Lab Instrument, Grödig, Australia). A sample is considered positive, if it gives an optical density of at least 0.1 and twice that of the negative control.

Chart No. 1

Record Solutions for the ELISA Test

Carbonate Buffer

Sodium carbonate Na_2CO_3.....1.59g

Sodium carbonate $NaHCO_3$.....2.93g

H_2O distilled up to.....1.00L

Sodium Acid NaN_3.....0.20g

Washing Solution

Concentrated solution 20X:

Sodium chloride NaCl.....146.29g

Trizma™/HCl.....39.40g

Tween™-20.....10.00ml

Thimerosal..... 1.00g

H_2O distilled up to.....1.00L

Phosphate Buffer (PBS)

NaCl 8.00g

KCL 0.20g

Na_2HPO_4.....1.15g

KH_2PO_4 .....0.20g

H_2O 1.00L

pH 7.3

Preparation of substrate

Substrate A

Sodium citrate.....15g

Citric acid.....6g

Urea peroxide.....1g

Distilled water up to.....1L

Substrate B (chromogenic substrate)

B-1 Tetramethyl benzidine (TMB).....200mg

Dimethylsulphoxide (DMSO).....250ml

B-2 sodium citrate..... 15mg

Citric acid.....6g

Distilled water.....1L

250ml of B-1 were mixed with 750ml of B-2.

In use, 50% of substrate A were mixed with 50% of substrate B.

Chart No. 1

(continued)

TRIS-casein 0.5%-Tween™ 0.05%

Concentrated solution 20X:.....50 ml

Distilled water milli-Q™ .....950 ml

Casein.....5 g

mix during 15 min.

adjust pH between 7.2 and 7.6 with NaOH 3N mix at 4°C until the next day allowing the solution be at room temperature ensure that the pH is between 7.2 and 7.6 otherwise adjusting it.

TRIS 50 mM pH 8.4

Trizma base ($C_4H_{11}NO_3$1;PM:121.1).....6.055 g

Thimersol (0.01%).....0.1 gr

Distilled water milli-Q™ .....1000 ml

adjust pH to 8.4 with HCl 3N

1.3 Purification and concentration of anti-BRSV polyclonal antibodies

Procedure for purifying affinity chromatography using a protein G module

The serum was diluted 1/5 with PBS and it was filtered on Millipore™ membrane having a porosity of 0.2µm at the same temperature as the purification took place and on the same day. The module 1500 of protein G was used. Many cycles had to be carried out. Each cycle comprises the following steps: loading, washing, eluting and rebalancing. The solutions used were: for loading, serum diluted 1/5 in PBS; for washing, PBS pH 7.4; for eluting, glycine-HCL 0.1M pH 2.5; for rebalancing, monobasic sodium phosphate 0.1M of pH 8. Once the elution obtained which is the purified serum, the pH was adjusted at 7 with TRIS 1M buffer.

Procedure for concentrating with a column of SP-Trisacryl

The elution obtained with the G protein was filtered with a Millipore™ membrane having a porosity of 0.2µm, at the same temperature as the concentration and on the same day. A column of 12cm by 18cm and 2L of SP-Trisacryl™ was used. The concentration cycle comprises: balancing with 50mM of sodium acetate pH 6; loading with the serum to be concentrated; rebalancing with 50mM of sodium acetate pH 6; elution with 50mM of sodium acetate pH 6, 200mM NaCl; rebalancing with 50mM of sodium acetate pH 6.

Dialysis in PBS

The sample was deposited in a dialysis membrane (Tubulaires Spectra/Por MWCO 10,000, Fisher, Montréal, Canada) which is deposited in a 10L beaker (Fisher) containing PBS and a magnetic bar (Fisher); reaction was allowed to take place for at least 48 h, while stirring, at 4°C. PBS was changed 3 times at the minimum.

Lyophilization

The purified and concentrated IgG were lyophilized in a Lio-San™ apparatus (Rolmex). The initial volume and the weight of the collected powder were noted in order to provide again in a water suspension for use. The polyclonal antibody thus treated is kept in sealed bags at -20°C.

1.4. Evaluation and verification of the bovine polyclonal antibody

Quantification of proteins

The BCA™ kit of Pierce (#23225) was used. For the preparation of the protein (STD) standard, STD and distilled water were mixed in equal proportions. Five plastic tubes were thereafter prepared as follows: a final volume of 100µl/tube and a concentration of 5 to 50µg/100µl were needed. Moreover, a control was prepared with 100µl of distilled water in a sixth tube.

Adequate dilutions of the samples were prepared, so that they can be found on the STD curve, always for a volume of 100µl.

Reagent B was mixed with reagent A 1:50; these two reagents are supplied in the kit. The mixture is well mixed and 2ml are added to each of the previously prepared tubes. The tubes were incubated during 30 min. at 60°C, and they are read with a Spectronic 1001 plus™ spectrophotometer, at 562nm (Milton Roy Spectronic, New York, USA). Thereafter, the curve is made with the controls and the optical density of the samples and their corresponding concentration of proteins are found.

Titration of the polyclonal antibody

The polyclonal antibody was titrated with ELISA by using the method described above in section I-1.2.

1.5. Results

Three lots of polyclonal antibodies have been produced.

Table 1

Production of polyclonal antibody

Lot Nº	Titer per 100µl*	Total product (L)
AL0692PRS-1	8100	0.5
AL0692PRS-2	8100	0.1
AL0692PRS-3	8100	0.1

* The titer represents the last dilution which gives an optical density at least twice higher than that of the negative control.

The lot designated AL0692PRS-1 was used. The other two lots, AL0692PRS-2 and AL0692PRS-3 were produced and used to verify the reproducibility of the method used.

2. Production of MAb

2.1. Preparation of MAb

Immunization of mice

BALB/c mice were inoculated by intraperitoneal injection with 250 µl of BRSV purified as described in section I-1.2, mixed with 250µl of incomplete Freund adjuvant, on days 0, 15 and 21. Three immunizations by utilizing only the purified virus were carried out on days -3, -2 and -1 before fusion.

Preparation of spleen cells

The preparation of spleen cells is carried out after euthanasia of the mice with CO_2, 24h after the last immunization. The mice are disinfected with ethanol alcohol 70% before removing the spleen. The spleen is deposited in a Petri box containing 5ml of the

medium Dulbecco's Modified Eagle Medium (DMEM rich in glucose, Gibco #430-2100EL) to which are added 3.7g/L of sodium bicarbonate, 4.8g/L of Hepes, 0.16g/L of sodium pyruvate, 2mM of L-glutamine and 0.2g/L of gentamicine. The spleen is dissected in order to free the cells. Then, the DMEM containing the cells is collected and the pieces of tissue are allowed to be decanted for 2 min. The supernatant is centrifuged at 250xg for 5 min. After two washings with DMEM, the cells are counted in DMEM.

Preparation of myeloma cells

NS-1 Ag4-1 (American Type Culture Collection, ATCC, 12301 Parklawn Drive, Rockville, MD 20852 USA, ATCC accession number TIB 18) myeloma cells are used as partner cells. The NS-1 Ag4-1 cells contain 65 chromosomes and synthesize the light kappa intracellular chain. The operation is carried out with cells in exponential growth phase and whose viability is at least 80%. They are counted after two washings in DMEM.

Fusion

10^7 myeloma cells and 10^8 spleen cells are mixed in DMEM. Then, a centrifugal operation was carried out for 5 min., at 500xg (Damon™\IEC HN-SII) and the supernatant is vacuumed.

1ml of polyethylene glycol 1450 (Baker) is added at a concentration of 37% by $1.6x10^8$ lymphocytes and the cells are again suspended during 1 min. 5ml of DMEM are added drop wise during 3 min., with a rotary movement. 5ml of DMEM containing 20 % of bovine fetal serum are added during 3 min., while still maintaining a rotary movement. Then, the volume may be completed to 50ml with DMEM containing 20 % of SFB.

Centrifugation was carried out during 5 min., at 500xg (Damon™/IEC HN-11) and the supernatant is vacuumed. The residue is resuspended in 20ml of DMEM containing 20% SFB. The cells are capped and 10^5 cells/wells are deposited in plates containing 96 wells (Corning™ 25860). Thereafter, 10^4 spleen cells from a normal mouse are added, in each well. After 24h, there are added 100µl/wells of DMEM-HAT 2X(DMEM to which there are added 10% of SFB, 27.22mg/L hypoxanthine, 7.56mg/L of thymidine and 1.52mg/L aminopterine).

Then, every third day, the medium is changed by adding DMEM-HAT 1X (DMEM to which there are added 10% SFB, 13.61mg/L of hyposanthine, 3,78mg/L of thymidine and 0.76mg/L aminopterine). By a daily microscopic examination, the wells where the cells are replicated are selected. 100µl thereof are collected, and are tested on screening ELISA. The positive samples were propagated in plates containing 24 wells (Corning™ 25820) and sub-cloned by the method of limit dilutions. The produced clones are again tested and they are frozen in liquid nitrogen if they appear to be positive.

Cells of clones secreting the desired MAb are incubated at least during three days with more than 80% confluence to permit the production of antibodies. The product is subject to centrifugation for 5 min., at 500xg (Damon™/IEC HN-SII). The supernatant obtained is used for all the subsequent steps.

Technique of screening ELISA

Plates prepared as indicated in section I-1.2 are used.

To test the selected hybridomas, or a MAb, 100µl of the sample are deposited in each of the wells and are incubated during 30 min. at 37°C. This is followed by three washings, with PBS-Tween™ and drying is carried out by inversion. There is then added in each

of the wells 100µl of anti-mouse goat antibody IgG (H+L), conjugated to peroxidase (Bio-Rad) diluted 1/4000. After a 30 min. incubation at 37°C, the product is washed three times with PBS-Tween™ and twice with the PBS. 100µl of chromogenic substrate mixture (50:50) are added in each well, the reaction is allowed to be carried out, for 10 min. at room temperature, and the reaction is stopped with 30µl of H_2SO_4 (4N).

Reading of the plate is made by bichromatism, at a wave length of 450nm with a reference of 550nm, on a spectrophotometer for ELISA (SLT Lab Instrument, Grödig, Australia). A sample was considered positive, if it gives a optical density which is higher than that of the control.

2.2. Characterization of the MAb

Type of immunoglobulin

The type of immunoglobulin of the MAb is determined by means of the INNO-LIA Mouse MAB Isotyping™ kit (Innogenetics, Belgium).

Technique of immunoblot

0.4ml of purified BRSV antigen and 0.4ml of the migration buffer (TRIS HCl 0.5 M pH 6.8, glycerol 10%, SDS 2%, bromophenol blue 0.2%) are mixed together and the mixture is boiled for 5 min. The migration is carried out on a polyacrylamide mini-gel, with a 4% concentration gel and a 10% migration gel.

After migration, a print of the gel is carried out on nitrocellulose paper using the buffer Tris 50mM pH 8.3, glycine 15mM and 20% methanol.

These nitrocellulose papers were incubated with a solution of PBS, 0.3% of fish gelatin (PBS-G) during 1 hour while steering, after which MAb diluted in PBS-G containing 0.05% Tween™ 20 are added and the mixture is incubated during 1.5 hours while steering. After washing with PBS, incubation is carried out in the presence

of anti-immunoglobulin mouse antibodies conjugated to peroxidase during 1 hour while steering.

After three washings with the PBS, chloronaphtol in methanol and PBS in the presence of peroxide are added. When the bands are visible, the reaction is stopped with water.

Neutralization test

50µl of each MAb are mixed with 100µl of BRSV containing 200 $DICT_{50}$ and the mixture is incubated during 1 h at 37°C. Plates containing 96 wells are used, which plates have flat bottoms (Corning™ 25860, Fisher), with confluent MDBK cells (see section I-1.1). 100µl/well of the antibody-virus are deposited in four wells. Incubation follows, during 1 h, at 37°C, in the presence of 2% of CO_2. Then, 100µl of a maintenance medium is added (Chart No. 2) in each well and incubation is allowed to proceed, during three days.

Verification of the cytopathogenic effect is carried out every day during 3 days. The presence of the cytopathic effect indicates that the MAb is not a neutralizing agent.

Chart No. 2

Preparation of Reagents for the Passage of Cells MDBK

Phosphate Buffer (PBS) pH 7.3

NaCl	8.00g
KCL	0.20g
Na_2HPO_4	1.15g
KH_2PO_4	0.20g
H_2O	1.00L

Culture Medium MEM

Minimum Essential Medium (MEM) (Gibco Laboratories #410-1100EE), to which there are added 2g/l of sodium bicarbonate, 10% bovine fetal serum (SFB) of Gibco, New York, USA, 1% of gentamicine 5mg/ml of Schering Canada, Pointe Claire, Quebec and 1% anti-PPLO 100X (pleuropneumonia-like organisms) of GIBCO Laboratories, New York, USA.

Maintenance Medium

MEM prepared as described above to which 2% of SFB is added.

Preparation of Trypsin EDTA 10X

Dissolving together 80g of sodium chloride (NaCl), 4g of potassium chloride (KCl), 10g of dextrose (D-Glucose) ($C_6H_{12}O_6$), 5.8g of sodium bicarbonate ($NaHCO_3$), 5g of trypsin (Difco 1/250) and 2g of versene, in 1L of distilled water. Adding 1ml of solution red phenol (0.5%) and 10ml of a mixture of penicillin-streptamycine. Filtration on Millipore™ membrane of 0.22µm porosity and adjusting the pH at 7.

2.3. Results

Eight MAb which are specific to BRSV have been developed and tested by ELISA using two RSV bovine strains (Q1 and VR794ATCC) and a strain of human origin as antigens. Two MAb, RSQ89C and RSQ90, reacted with all the strains used. Considering that the MAb RSQ89C has given the higher optical density, it was selected as second antibody to develop the double sandwich ELISA.

The MAb RSQ89C presents a type of immunoglobulin IgG1 and appears non-neutralizing.

Fig. 1 shows that the MAb RSQ89C reacts with nucleoprotein.

Three different lots of the MAb RSQ89C have been produced and frozen at -20°C. The lot designated as SE2192M89C was used during the entire project. Two other lots, AL1194M89C and AL2494M89C have been produced and used to verify the reproducibility of the method used.

Table 2

Production of the MAb

Lot	Volume (L)
SE2192M89C	0.65
AL1194M89C	0.11
AL2694M89C	0.08

ANTIGENS USED IN THE ELISA KIT

3. Cells of bovine kidneys (MDBK)

3.1. Passages of the cells of bovine kidneys (MDBK)

The cellular division is carried out every third to fourth day. After having proceeded to a microscopic examination of the cellular monolayer and having observed that the MDBK cells are really confluent, the culture flask is rinsed (Corning, Pointe Claire,

Canada) twice with PBS in order to remove all trace of serum which would risk to inactivate trypsin-EDTA. The cellular monolayer is rinsed twice with trypsin-EDTA (Chart No. 2) having a final dilution of 0.25%. Trypsin-EDTA is allowed to react, during 3 to 10 minutes, at 37°C. The flask is knocked to remove the cells and then a medium MEM is added (Chart No. 2). The cells thus removed are passed through a needle 23Gl™ (Becton Dickinson, Franklin Lakes NJ, #305145). Thereafter, they are distributed at the rate of 60,000 cells per cm^2 on a flat bottom plate having 96 wells (Corning™ 25860, Fisher), or at the rate of 50,000 cells per cm^2 in a $150cm^2$ flask (Corning™ 25120, Fisher). The flask is filled to 40 ml with MEM (Chart No. 2). The cells are incubated at 37°C.

3.2. Control of the quality of the MDBK cells

One a month, the cells which are present are tested by the ELISA technique (Laboratoire de Diagnostique, Virologie, Faculté de médecine vétérinaire) to make sure that there is no bovine viral diarrhea virus.

4. Production of positive control

4.1. The virus

The BRSV virus used is the Quebec strain Q1 isolated and characterized by Dr. Y. Elazhary (1980, *Can. J. Comp. Med.*, **44**:299-303). The strain has been identified as virus BRSV by the neutralization test. The virus which is inoculated on the cells is at passage number 20.

4.2 Inoculation of cells with the virus

MDBK cells which are confluent at 70-90% and are 24 to 48 hours old are used. Their culture medium is removed by washing with PBS. An adequate dilution of virus is thereafter added, which is made in the mainte-

nance medium (Chart No. 2) to have a multiplicity of infection of 0.005 to 0.1 (10ml for a 150cm^2).

The reaction is allowed to proceed during 1h, at 37°C, while stirring. The volume is then completed, depending on the flask used, with a maintenance medium. Incubation is carried out at 37°C.

4.3. Collection of the virus

The inoculated cells are observed daily to detect the cytopathic effect. When the latter reaches 50% of the cells, the virus is collected. It is collected by means of a policeman and it is frozen at -70°C.

4.4. Verification of the virus produced

Titration of virus on cells MDBK

There are used plates of 96 wells, prepared with the MDBK cells. Dilution of the virus are made with factors of 10, in the maintenance medium, and there are deposited 100µl/well of each dilution, in four wells. Incubation is carried out during 1h, at 37°C, in the presence of 2% of CO_2. Then, 100µl of maintenance medium is added in each well and incubation is allowed to proceed during three days.

The verification of the cytopathic effect is carried out every day and the viral titter is calculated according to the Kärber formula.

4.5. Results

Three lots of BRSV have been produced and preserved at -70°C. The lots designated as AL3092VRS and OE1292VRS were used all along this project. The lot AL0294VRS was used to verify the reproducibility of the method.

Table 3

Production of positive control

Lot	Volume (ml)	Titer
AL3092VRS	200	$10^4 DICT_{50}/ml$
OE1292VRS	100	$10^{5.5} DICT_{50}/ml$
AL0294VRS	100	$10^{5.5} DICT_{50}/ml$

5. Production of the negative control antigen

Trypsinization of 3 days old cells is carried out as described in section I-3.1. The cells are resuspended in the maintenance medium at the rate of 250,000 cells per ml and frozen at -70°C.

5.1 Results

Three lots of the negative control were produced and preserved at -70°C. The lot designated at AL1192CMDBK was used all along this project. The lots MI2194CMDBK and MI2594CMDBK were used to verify the reproducibility of the method.

Table 4

Production of negative control

Lot Nº	Volume (ml)
AL1192CMDBK	200
MI2194CMDBK	300
MI2594CMDBK	200

II- STANDARDIZATION OF THE ELISA TEST TO DETECT BRSV

1. Determination of the dilution of the polyclonal antibody

The objective was to determine the dilution of the polyclonal antibody to be used for the sensibilization of the plates.

Concave bottom plate with 96 wells, made of polystyrene Maxisorp™ U16 (Nunc) were used. The lyophilized polyclonal antibody lot AL0692PRS-1 was

resuspended at the rate of 0.0147mg of powder/ml of distilled water. The thus resuspended polyclonal antibody contains 4.28mg of protein/ml. It was diluted in PBS at the following concentrations: 8.5, 4.2, 2.1, 1.0, 0.5 and 0.2µg of protein/100µl. These dilutions were used to coat the wells at the rate of 100µl/well. After a 1h incubation at 37°C and 3 washings with the washing solution (Chart No. 1), the plates were blocked with TRIS, casein 0.5%, Tween™ 0.05% (TRIS-C-T, Chart No. 1) during 30 min. at 37°C. The BRSV lot #AL3092VRS diluted 1/2 in TRIS-C-T was incubated during 15 min. at room temperature in the presence of the MAb RSQ89C diluted 1/2 in TRIS-C-T. The mixture of virus and MAb was added to the plate at the rate of 100µl/well. After 5 min. of incubation at room temperature and 3 washings, the anti-mouse antibody conjugated to peroxidase (Jackson ImmunoResearch Laboratories, West Grove, USA) diluted 1/2000 in PBS was added at the rate of 100µl/well and incubated 30 min. at room temperature. After 3 washings, 100µl of chromogenic substrate were added in each well. After 10 min., the reaction was stopped with 30µl of H_2SO_4 4N. The plate is read by bichromatism, at a wave length of 450nm with a reference of 550nm, on a spectrophotometer for ELISA (SLT Lab Instrument, Grödig, Australia).

In Fig. 2, the values of optical density are expressed as the ratio of the optical density of each dilution of the polyclonal antibody. The bars represent the standard deviation of two experiments. The results, illustrated in Fig. 2, establish that the maximum reactivity was reached with a concentration of 1µg/well. This represents a working dilution of 1/400.

2. Determination of the dilution of the MAb RSQ89C

The objective was to determine the working dilution of the MAb RSQ89C Lot #SE2192M89C. This lot of MAb was used during the entire project.

An ELISA test as described above in section II-1 was carried out. The polyclonal antibody was used at the rate of 1µg/well and the BRSV diluted 1/10. The dilutions per factor of 2, 1/4 to 1/64 of MAb were tested. The working dilution was defined as the last dilution giving the highest optical density.

Table 5

Determination of the working dilution of the MAb

	1/4*	1/8	1/16	1/64
(+) Control Lot #AL3092VRS	1.42**	1.40	1.28	0.85
(-) Control Lot #AL1192CM	0.10	0.10	0.09	0.09

* Dilutions of the MAb RSQ89C
**Optical densities (average of two wells) detected by using different dilutions of the MAb RSQ89C

The results of this experiment indicate that the working dilution of the MAb RSQ89C was 1/8.

3. The anti-mouse antibody labeled with peroxidase

The objective was to determine the working dilution of the anti-mouse antibody labeled with peroxidase (conjugated).

The anti-mouse antibody labeled with peroxidase produced by Jackson ImmunoResearch Laboratories, West Grove, USA was used (lot #115-030-061). The ELISA test was carried out as described in section II-1. The plate was sensitized with different concentrations of the polyclonal antibody. The conjugate was diluted 1/1000, 1/3000, 1/4000 and 1/8000.

Table 6

Determination of the conjugate working dilution

Polyclonal antibody dilution	Antigen	Conjugate dilution			
		1/1000	1/3000	1/4000	1/8000
1/50	Control +	1.93	1.68	1.12	0.64
1/50	Control -	0.26*	0.16	0.07	0.04
1/200	Control +	1.77	1.43	1.01	0.55
1/200	Control -	0.17	0.12	0.05	0.02
1/400	Control +	1.62	1.13	0.83	0.41
1/400	Control -	0.14	0.09	0.04	0.02

*Dilution of the polyclonal antibody
*Optical densities (average of two wells)

The best ratio between the optical density of the positive control and that of the negative control was detected at a dilution 1/400 of the polyclonal antibody and at a dilution 1/3000 of the conjugate. The working dilution was established at 1/3000.

The lot of conjugate No. 115-035-062 was titrated and used during the entire project. Its working dilution was established in function of the optical densities obtained with the first lot of the conjugate used (#115-030-061).

Table 7

Determination of the working dilution of conjugate Lot #115-035-062

Antigen	Conjugate dilution Lot # 115-030-061			Conjugate dilution Lot #115-035-062		
	1/1000	1/3000	1/4000	1/1000	1/3000	1/4000
Control +	1.48*	1.30	0.97	1.52	1.37	1.06
Control -	0.24	0.15	0.07	0.23	0.15	0.07

*Optical densities (average of two wells)

A dilution of 1/3000 was selected. The immunological sensitivity of virus detection previously found was verified with the new dilution factor of the conjugate before adopting it.

4. Comparison of two ELISA methods with and without a pre-incubation of the antigen and the MAb

The objective was to compare two ELISA methods: with and without a pre-incubation of the antigen and the MAb.

The optical densities obtained by using the positive control Lot #AL3092VRS diluted 1/10 by the two methods were compared. The ELISA test with a pre-incubation of the antigen and the MAb was carried as described in section II-1. During the second test, the antigen and the MAb were added in to subsequent steps and each were incubated during 15 min. at room temperature.

Table 8

Method of incubation of the antigen and the MAb

Method without pre-incubation of antigen and MAb	Method with pre-incubation of antigen and MAb
1.64 ± 0.23*	1.22 ± 0.33

* The given optical densities represent the average and the standard deviation of three different experiments. The background noise was removed from the given values of optical density.

The method without pre-incubation of the antigen and the MAb was selected.

5. Determination of the incubation time for each of the ELISA steps

The objective was to establish the time of incubation for each of the steps of the ELISA.

The plates were sensitized with the polyclonal antibody at the rate of 1µg/well during 1 hour at 37°C. After blocking with the TRIS-C-T during 30 min. at 37°C, the positive control diluted 10^{-1} and 10^{-2} was

added. The MAb was diluted 1/8 and the conjugate 1/3000.

During the determination of the time of incubation of the antigen, the latter was incubated during 5, 15, 30 and 45 min., the MAb was incubated during 15 min. and the conjugate during 30 min. The incubations were carried out at room temperature. The strongest reaction was detected when the antigen was incubated during 45 min.

During the determination of the time of incubation of the MAb, the antigen was incubated during 45 min., the MAb during 5, 15, 30 and 45 min. and the conjugate during 30 min. The incubations were carried out at room temperature. The strongest reaction was detected when the MAb was incubated during 5 min.

During the determination of the time of incubation of the conjugate, the antigen was incubated during 45 min., the MAb during 5 min. and the conjugate during 5, 15, 30 and 45 min. The incubations were carried at room temperature. The strongest reaction was detected when the conjugate was incubated during 30 min.

6. Conclusion of the standardization of the ELISA test to detect BRSV

Final protocol of the ELISA test

1- The round bottom plates with 96 wells, of polystyrene Maxisorp™ U16 (Nunc) were used. The lyophilized polyclonal antibody lot AL0692PRS-1 was resuspended at the rate of 0.0147mg of powder/ml distilled water. The polyclonal antibody thus resuspended contains 4.28mg of protein per ml. It was diluted 1/400 in PBS, resulting in a concentration of 1µg of protein per 100µl. This dilution was used to sensitized the wells at the rate of 100µl/well. An incubation of 1 h at 37°C was used.

2- After 3 washings with the washing solution (Chart No. 1), the plates were blocked with TRIS-C-T during 30 min. at 37°C.

3- After 3 washings, 100µl/well of antigen was added and incubated during 45 min. at room temperature.

4- After 3 washings, the MAb RSQ89C lot No. SE2192M89C diluted 1/8 in TRIS at pH 8.4 was added at the rate of 100µl/well and incubated during 5 min. at room temperature.

5- After 3 washings, the conjugate lot No. 115-035-062 diluted 1/3000 in TRIS-C-T was added at the rate of 100µl/well and incubated during 30 min. at room temperature.

6- After 3 washings, the developing solution (chromogenic substrate) is added at the rate of 100µl/well.

7- After 10 min., the development of a blue color was noted if the reading is visual. When the reading is made with a spectrophotometer, 30µl/well of stopping solution are added.

This protocol was used during all subsequent steps.

III- EVALUATION OF ELISA

1. Panel of antigens

The objective was to prepare a panel of reference antigens made of two strongly positive samples, two weakly positive and two negative, in order to use it for the validation of the reagents and the kits.

The antigen panel is made of 6 samples:

HighR1: RSV of lot OE1292VRS non diluted
HighR2: RSV of lot ALO294VRS non diluted
LowR1: RSV of lot OE1292VRS diluted 1/20
LowR2: RSV of lot ALO294VRS diluted 1/60
NegR1: Negative control lot # MI2194CMDBK
NegR2: Negative control lot # MI2594CMDBK

Each antigen was kept in a 20 aliquots of 1 ml and frozen at -70°C.

Each antigen was tested in 32 wells on 5 plates (at the rate of 8 or 4 wells per plate) independently test by two operators. The average optical density (O.D.), as well as the standard deviation, the percentage of the standard deviation and the lower and upper limits of acceptance were calculated for each antigen.

The lower and upper limits of acceptance have been established as described by Wright, P. (1987, *Vet. Immunol. Immunopathol.*, **17**:441-452):

Average of O.D. + (3 x standard deviation) = upper limit of acceptance;

Average of O.D. - (3 x standard deviation) = lower limit of acceptance.

Table 9

Panel of antigens

Antigen	O.D. Mean	Standard deviation	Deviation %	Minimum O.D. value	Maximum O.D. value
HighR1	1.628	0.109	6.72	1.301	1.955
HighR2	1.780	0.136	7.64	1.372	2.188
LowR1	0.533	0.051	9.71	0.380	0.686
LowR2	0.447	0.041	9.17	0.324	0.570
NegR1	0.077	0.015	20.25	0.032	0.122
NegR2	0.081	0.025	31.35	0.006	0.156

Table 9 shows that the percentage of standard deviation of the positive antigens (strong and weak) in all case is lower than 10. Consequently, the noted variation is acceptable since a percentage of standard deviation of 15% is recommended as limit of acceptance (Gall, D. et al., 1992, *Guidelines for the development, optimization and standardization of the targeting ELISA procedure*, ADRI, Nepean). This value may not be considered in the case of negative antigens, since very

small variations may represent a very high deviation percentage.

The lower and upper O.D. limits indicate the limits between which the optical density values must be found each time the panel of antigen will be used so that the test be validated.

Moreover, the average of the optical density, as well as the standard deviation, the percentage of standard deviation and the lower and upper limits of acceptance have been calculated for the positive (BRSV lot #AL3092VRS and negative (cells MDBK Lot #AL1192CMDBK) controls used all along the evaluation of the developed ELISA test. The two controls were diluted 1/5 in TRIS 50mM pH 8.4.

Table 10

Positive (C+) and negative (C-) controls

Antigen	O.D. Mean	Standard deviation	Deviation %	Minimum O.D. value	Maximum O.D. value
C+	1.357	0.176	12.9	0.829	1.885
C-	0.072	0.025	34	0.003	0.147

Table 10 shows the lower and upper O.D. limits between which the optical density values should be found each time C+ and C- will be used.

2. Variation intra-plate

The objective was to verify if the localization of the sample on the plate had an influence on the optical densities obtained.

Each sample from which the panel of antigen described above was made as well as the C+ and C- have been tested on 8 wells situated in 4 different locations of the plate (Table 11).

Table 11

Distribution of the panel of antigen on the plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	C+	C+			Neg R2	Neg R2	C+	C+			Neg R2	Neg R2
B	C-	C-			Low R2	Low R2	C-	C-			Low R2	Low R2
C	High R1	High R1			High R2	High R2	High R1	High R1			High R2	High R2
D	Low R1	Low R1			Neg R1	Neg R1	Low R1	Low R1			Neg R1	Neg R1
E	Neg R1	Neg R1			Low R1	Low R1	Neg R1	Neg R1			Low R1	Low R1
F	High R2	High R2			High R1	High R1	High R2	High R2			High R1	High R1
G	Low R2	Low R2			C-	C-	Low R2	Low R2			C-	C-
H	Neg R2	Neg R2			C+	C+	Neg R2	Neg R2			C+	C+

The results obtained on 5 plates are expressed depending on the optical density average, the standard deviation and the percentage of spread in Tables 12 to 16.

Table 12

Plate No. 1

Antigen	Mean	Standard deviation	Deviation %
HighR1	1.566	0.080	5.14
HighR2	1.704	0.090	5.28
LowR1	0.528	0.045	8.52
LowR2	0.480	0.059	12.46
NegR1	0.070	0.004	7.00
NegR2	0.076	0.009	11.8
C+	1.578	0.071	4.51
C-	0.074	0.006	8.89

Table 13

Plate No. 2

Antigen	Mean	Standard deviation	Deviation %
HighR1	1.619	0.057	3.55
HighR2	1.751	0.060	3.45
LowR1	0.578	0.037	6.44
LowR2	0.440	0.025	5.75
NegR1	0.067	0.005	7.42
NegR2	0.066	0.005	8.76
C+	1.631	0.032	1.96
C-	0.062	0.004	6.37

Table 14

Plate No. 3

Antigen	Mean	Standard deviation	Deviation %
HighR1	1.755	0.098	5.58
HighR2	1.914	0.096	5.03
LowR1	0.541	0.036	6.75
LowR2	0.444	0.017	3.94
NegR1	0.075	0.007	10.02
NegR2	0.074	0.004	6.57
C+	1.768	0.118	6.70
C-	0.071	0.004	6.70

Table 15

Plate No. 4

Antigen	Mean	Standard deviation	Deviation %
HighR1	1.644	0.059	3.60
HighR2	1.883	0.142	7.58
LowR1	0.469	0.018	3.83
LowR2	0.436	0.031	7.30
NegR1	0.087	0.014	16.50
NegR2	0.099	0.047	47.57
C+	1.661	0.108	6.51
C-	0.088	0.030	35.00

Table 16

Plate No. 5

Antigen	Mean	Standard deviation	Deviation %
HighR1	1.504	0.028	1.8
HighR2	1.617	0.088	5.4
LowR1	0.498	0.059	11.8
LowR2	0.416	0.036	8.7
NegR1	0.105	0.022	21.1
NegR2	0.114	0.037	32.8
C+	1.563	0.054	3.4
C-	0.095	0.023	24.0

Tables 12 to 16 show that the intra-plate variation is acceptable since the standard deviation percentage, for all the positive antigens of each plate, was in all cases lower than 15%. Therefore, the results obtained with the samples are independent of the localization on the plate.

3. Variation inter-plates

The objective was to determine the variation of optical densities obtained with the same sample tested on different plates.

The optical density averages given in Tables 12 to 16 were compared. The average of these averages was calculated as well as the standard deviation and the percentage of deviation (Table 17).

Table 17

Variation Inter-Plate

Antigen	Mean	Standard deviation	% deviation
HighR1	1.617	0.093	5.79
HighR2	1.773	0.124	6.99
LowR1	0.523	0.041	7.99
LowR2	0.443	0.023	5.19
NegR1	0.081	0.015	18.88
NegR2	0.085	0.019	23.18
C+	1.640	0.081	4.97
C-	0.078	0.013	17.05

Table 17 shows that the variation inter-plate is acceptable since the percentage of standard deviation for all the positive antigens was in all cases lower than 15%. Therefore, the results obtained with the samples are reproducible on different plates.

4. Immunological Sensitivity of ELISA

The objective was to test the immunological sensitivity of the ELISA test developed.

In order to determine viral titer that the ELISA test could detect, an ELISA test was provided by using BRSV as antigen at different dilutions (10^{-1}, 10^{-2} and 10^{-3}). The BRSV used a titer of $10^4 DICT_{50}/ml$.

The results have shown that the last dilution of positive virus was 10^{-2}, therefore the ELISA test has detected up to $10^2 DICT_{50}/ml$ of BRSV.

5. Immunological Specificity of ELISA

The objective was to determine the immunological specificity of ELISA.

The ELISA was made by utilizing the following viral strains as antigens: bovine adenovirus; equine viral arteritis; bovine herpes virus type 4; bovine viral diarrhea virus; bovine coronavirus; feline leukemia virus; virus of infectious bovine rhinotracheitis; equine influenza virus; bovine parainfluenza virus; canine parvovirus; bovine rotavirus; purified bovine syncytial respiratory virus Q1; bovine syncytial respiratory virus VR794 ATCC; human syncytial respiratory virus; bovine syncytial respiratory virus Q1.

The results have shown that the ELISA test detects only the strains of human RSV virus and the bovine strains: Q1 and VR794 ATCC (Fig. 3), therefore it is specific to RSV.

6. Relative sensitivity and specificity of the ELISA

In order to evaluate the sensitivity and specificity of the ELISA test for the detection of the bovine RSV antigen, two different experiments were conducted. The first experiment consists in the detection of bovine RSV antigen in the nasal secretions of calf infected experimentally, while the second experiment consists in the detection of bovine RSV antigen in the nasal secretions of calf of different herds.

6.1 Description of the Experiments

6.1.1 Alternative: Experimental Infection

Holstein calves two weeks old were used. Four animals which have shown to be negative towards antibodies and the following virus: bovine rhinothracheitis (IBR), bovine viral diarrhea (BVD), parainfluenza 3 (PL_3) and bovine respiratory syncytial virus (BRSV) have been selected.

Among these four calves, two have been infected by intranasal way through a respiratory mask containing the nose and the mouth. A dose of $10^6 DICT_{50}$ in a volume of 5 ml has been given to each calf. The strain used was the American strain BRSV(CA-1) isolated during an epidemic outbreak in California. The two other calves have not been infected and were used as control. The nasal secretions have been collected daily and tested for the presence of the respiratory syncytial virus by viral isolation on MDBK cells and by the ELISA technique. The clinical signs have also been taken out. A calf is considered as having clinical signs if it presents a high temperature, nasal run or an increase of the breathing rhythm.

6.1.2 Alternative: Samples in the Field

Nasal secretions of 864 calves 1 to 12 weeks old have been collected in 17 farms. These samples were tested for the presence of the BRSV virus simultane-

ously by the two methods indicated above. On these calves the clinical signs were not taken into consideration because the samples were taken up randomly in the farms.

6.2 Description of the Techniques

6.2.1 Viral Isolation and Detection of BRSV

The MDBK cells were distributed in plates of 24 wells as described in point I-3.1. After 3 hours, while the cells are 50% confluent, the growth medium was removed and the cells were washed with MEM containing 1% antibiotic. Each sample of nasal secretions is inoculated on two wells, 200 µl per well. Previously, the nasal secretions have been filtered on 0.45 µm. For each plate, a maintenance medium (Chart No. 2) is used as negative control and BRSV virus diluted 1/10 in this medium is used as positive control. The plates are incubated during 1 hour at 37°C with 2% CO_2. Then, 1 ml of the maintenance medium is added in each well. The plates were examined daily during four days and if necessary a second passage is carried out.

After four days, the plates are fixed with formaldehyde. In each well, 1 ml of formaldehyde diluted 1:7 in distilled water is put in. After 10 minutes of incubation at room temperature, the cells were washed 3 times with PBS-Tween™. A solution of 5% skim milk in PBS is deposited on the cells, 250 µl per well and incubated 30 minutes at 37°C. Then, the cells are washed 3 times with PBS-Tween™ and the MAb 89c is introduced, 250 µl per well, and incubated 60 minutes at 37°C. The cells are again washed 3 times and at 250 µl of anti-mouse conjugated peroxidase, produced on goat, diluted 1/200 in a solution of PBS-0.1% Tween™-1% gelatin are placed in each well. The plates are incubated 90 minutes at 37°C. The cells are washed 3 times and 250 µl per wells of substrate 3-Amino-9-Ethylcarbo-

zole (AEC) is applied. After a 10 min. incubation followed by 3 last washings with PBS-Tween™, the plates are dried and a reading is carried out on an inversion microscope with a small objective 10X. The presence of a brownish color in the cytoplasm indicates the presence of virus.

6.3 Results

6.3.1 Alternative: Experimental Infection

In Table 18, the results of a viral isolation, ELISA and clinical signs are found. A first analysis demonstrates that the presence of the antigen is detected already with the appearance of clinical signs and that the antigen can no more be detected with the disappearance of these signs. On a comparative point of view, all samples which are positive with viral isolation are also positive with ELISA, and therefore there is concordance between the two methods.

Table 18
Comparison of ELISA and viral isolation on infected and non-infected calves

	INFECTED CALVES						CONTROL CALVES					
	CALF 1			CALF 2			CALF 3			CALF 4		
Sampling day	Isolated	ELISA	Signs	Isolated	ELISA	Signs	Isolated	ELISA	Signs	Isolated	ELISA	Signs
DAY 1	na	na	-	-	- 0,016*	-	-	na	-	-	- 0,044	-
DAY 2	-	- 0,039	-	-	- 0,021	-	-	- 0,010	-	-	- 0,038	-
DAY 3	-	- 0,022	-	-	- 0,043	-	-	- 0,015	-	-	- 0,021	-
DAY 4	-	- 0,065	-	-	- 0,042	-	-	- 0,021	-	-	- 0,014	-
DAY 5	na	na	-	+	+ 0,218	+	-	na	-	-	- 0,032	-
DAY 6	na	na	-	+	+ 0,503	+	-	na	-	-	- 0,027	-
DAY 7	+	+ 0,760	+	+	+ 0,437	+	-	- 0,017	-	-	- 0,035	-
DAY 8	+	+ 0,144	+	+	+ 0,524	+	-	- 0,021	-	-	- 0,046	-
DAY 9	-	- 0,034	-	+	+ 0,396	+	-	- 0,028	-	-	- 0,024	-
DAY 10	na	na	-	-	- 0,031	-	-	na	-	-	- 0,032	-

na: nasal secretions not available
Isolated: viral isolation
*: optical density

6.3.2 Alternative: Samples in the field

In Table 19, the distribution of the samples of different farms is found. Some samples could not be analyzed by viral isolation because of a lack of nasal secretion volume. Therefore, these samples were excluded during the determination of the sensitivity and the specificity which will enable the calculation of the limit value (cut off) of the ELISA test. This limit value will serve to determine if a sample is positive or not in the ELISA test. By removing these samples, we have a total of 857 specimens which have been analyzed by the two methods.

Table 19

Distribution of the samples in the farms and results of the analysis

FARMS	Nb tests	ELISA (+)	ISOLATED (+)	ELISA (-)	ISOLATED (-)	ISOLATED na
FARM 1	50	0	0	50	50	0
FARM 2	20	4	3	16	16	1
FARM 3	24	0	0	24	24	0
FARM 4	91	3	3	88	88	0
FARM 5	73	2	1	71	71	1
FARM 6	2	0	0	2	2	0
FARM 7	6	0	0	6	6	0
FARM 8	6	0	0	6	6	0
FARM 9	13	2	1	11	11	1
FARM 10	24	1	1	23	23	0
FARM 11	19	0	0	19	19	0
FARM 12	3	0	0	3	3	0
FARM 13	372	10	8	362	362	2
FARM 14	30	0	0	30	30	0
FARM 15	68	2	1	66	66	1
FARM 16	47	0	0	47	47	0
FARM 17	16	7	7	9	8	1
TOTAL	864	31	25	833	832	7

ISOLATED na: samples not available for viral isolation

The same samples are positive with viral isolation and ELISA.

Table 20

Limit and average values of the negative controls (NC) of the entire ELISA plates

Average of negative controls (NC) of all the plates:	0.062
Standard deviation C of negative controls (NC)of all the plates:	0.014
Maximum value of negative controls:	0.093
Minimum value of negative controls:	0.032

Considering that the viral isolation test is the test of reference, the relative sensitivity (Se) and the relative specificity (Sp) have been calculated

and the correlation between the two tests, viral isolation and ELISA was measured by the kappa factor (K).

In order to determine the limit value (cutoff), two factors were considered. First, the value of the negative control and second the ratio (SAM_{od}/NC_{od}) between the optical density of the sample and the optical density of the negative control. This way of operating enables to account for the operator and also of the specific values of each plate.

Three different thresholds for the value of the negative control (0.09; 0.10; 0.11) have been used to calculate the relative sensitivity and specificity with SAM_{od}/NC_{od} ratios between 1 and 2 (Table 21). The first threshold tested corresponds to the maximum value of the negative controls of all the plates which have been carried out (Table 20).

It can be observed that the limit threshold of 0.100 is the threshold which enables to have a maximum and sensitivity and specificity (100%).

Speaking of this threshold of 0.100, it will be noted that we have 100% sensitivity and specificity for the entire range of ratio between 1 and 1.5. In order to find the exact ratio, 6 kits have been prepared and the test was made by 6 operators having little or no experience with ELISA (results; Table 22). It appears that the ratio 1.5 really establishes the limit since none of the operators has exceeded this value with the negative controls. However, with operator E, a ratio of 1.494 is obtained. This value is very close to 1.5, and it is therefor concluded that the ratios between 1.5 and 1.7 should be considered as doubtful.

Thus, the interpretation of the results could be summarized as follows:

- if the optical density of a sample is lower than 0.100, this sample is negative.
- if the optical density of a sample is higher or equal to 0.100 and the ratio (SAM_{od}/NC_{od}) is smaller than 1.5, than the sample is negative.
- if the optical density of a sample is higher or equal to 0.100 and the ratio (SAM_{od}/NC_{od}) is between 1.5 and 1.7 then the sample is doubtful and the test should be taken over again.
- if the optical density of a sample is higher or equal to 0.100 and the ratio (SAM_{od}/NC_{od}) is higher or equal to 1.7 then the sample is positive.

By considering this interpretation, the relative sensitivity of the test is 92%, the specificity is 100% and the kappa factor is 0.957.

Table 21

Variation of the ratio of the optical density of the samples (SAM_{od}) on the optical density of the negative control (NC_{od}) as a function of a minimum limit threshold (optical density of the negative control)

RATIO SAM_{od} / NC_{od}	ISOL. C + ELISA +	ISOL.C + ELISA -	ISOL.C - ELISA +	ISOL.C - ELISA -	SENSITIVITY	SPECIFICITY	CONCOR-DANCE	KAPPA
MINIMUM THRESHOLD 0,090								
1,0	25	0	5	827	1,000	0,994	0,994	0,906
1,1	25	0	5	827	1,000	0,994	0,994	0,906
1,2	25	0	2	830	1,000	0,998	0,998	0,960
1,3	25	0	1	831	1,000	0,999	0,999	0,980
1,4	25	0	1	831	1,000	0,999	0,999	0,980
1,5	25	0	1	831	1,000	0,999	0,999	0,980
1,6	24	1	1	831	0,960	0,999	0,998	0,959
1,7	23	2	1	831	0,920	0,999	0,996	0,937
1,8	21	4	1	831	0,840	0,999	0,994	0,891
1,9	20	5	1	831	0,800	0,999	0,993	0,866
2,0	20	5	1	831	0,800	0,999	0,993	0,866
MINIMUM THRESHOLD 0,100								
1,0	25	0	0	832	1,000	1,000	1,000	1,000
1,1	25	0	0	832	1,000	1,000	1,000	1,000
1,2	25	0	0	832	1,000	1,000	1,000	1,000
1,3	25	0	0	832	1,000	1,000	1,000	1,000
1,4	25	0	0	832	1,000	1,000	1,000	1,000
1,5	25	0	0	832	1,000	1,000	1,000	1,000
1,6	24	1	0	832	0,960	1,000	0,999	0,979
1,7	23	2	0	832	0,920	1,000	0,998	0,957
1,8	21	4	0	832	0,840	1,000	0,995	0,911
1,9	20	5	0	832	0,800	1,000	0,994	0,886
2,0	20	5	0	832	0,800	1,000	0,994	0,886
MINIMUM THRESHOLD 0,110								
1,0	23	2	0	832	0,920	1,000	0,998	0,957
1,1	23	2	0	832	0,920	1,000	0,998	0,957
1,2	23	2	0	832	0,920	1,000	0,998	0,957
1,3	23	2	0	832	0,920	1,000	0,998	0,957
1,4	23	2	0	832	0,920	1,000	0,998	0,957
1,5	23	2	0	832	0,920	1,000	0,998	0,957
1,6	23	2	0	832	0,920	1,000	0,998	0,957
1,7	23	2	0	832	0,920	1,000	0,998	0,957
1,8	21	4	0	832	0,840	1,000	0,995	0,911
1,9	20	5	0	832	0,800	1,000	0,994	0,886
2,0	20	5	0	832	0,800	1,000	0,994	0,886

Table 22

Studies of the detection of the negative samples by different operators

	MANIPULATORS					
	A	B	C	D	E	F
Negative control	0,114	0,085	0,102	0,085	0,075	0,112
Negative sample 1	0,114	0,099	0,118	0,126	0,087	0,091
Negative sample 2	0,117	0,110	0,115	0,127	0,102	0,096
Ratio 1*	1,000	1,165	1,157	1,482	1,160	0,813
Ratio 2	1,03	1,294	1,127	1,494	1,360	0,857

Ratio 1: Optical density sample 1/ optical density of negative control
Ratio 2: Optical density sample 2/ optical density of negative control

7. Correlation between visual reading and reading with spectrophotometer

The correlation between visual reading and reading was established with spectrophotometer.

The visual reading and the reading with spectrophotometer was realized on 653 different wells (Table 23). Visual reading was carried out at least by two persons. In all cases the reading obtained by different persons was the same.

Table 23

Visual Reading and Reading with Spectrophotometer

Optical density values	Visual Reading		spectrophotometer Reading		Total
	negative	positive	negative	positive	
≥0.2	0*	304	0	304	304
0.18 <0.2	2	10	0	12	12
0.16 <0.18	0	3	0	3	3
0.14 <0.16	5	0	0	5	5
0.12 <0.14	1	1	1	1	2
0.10 <0.12	4	0	4	0	4
<0.1	323	0	323	0	323
Total	335	318	328	325	653

*Number of samples

Table 23 shows that the visual reading 100% correlation with the spectrophotometer when the optical density is higher than 0.2 or less than 0.1. When the optical density is between 0.1 and 0.2, the specificity and sensitivity of the visual reading towards spectrophotometer reading are 100 and 67% respectively. It can be concluded that the specificity of the visual reading is very good in all cases. On the other hand, its sensitivity is smaller than that of the spectrophotometer reading, when the optical densities of positive samples are between 0.1 and 0.2.

8. Reproducibility of the production of polyclonal antibodies

The reproducibility of the method of purification of polyclonal antibodies was tested.

The polyclonal antibodies lot #AL0692PRS-2 was purified according to the method described in section I-1.3. Its working dilution was established at 1/400 according to the methodology described in section II-1. The ELISA test was carried out by utilizing the polyclonal antibody purified diluted 1/400 in order to sensitize the plates. The panel of antigen (section III-1) as well as the C+ and the C-, have been used as antigens. A sensitized plate with the polyclonal antibody lot #Av0692PRS-1 was used as a reference plate.

Table 24

Reproducibility of the purification of polyclonal antibodies

Antigens	Polyclonal Antibodies	
	Lot #AL0692PRS-1	Lot #AL0692PRS-2
C +	1.230 (1.6)*	1.303 (1.4)
C -	0.047 (1.5)	0.074 (18)
HighR1	1.287 (2.6)	1.395 (5.4)
HighR2	1.490 (2.9)	1.798 (3.8)
LowR1	0.464 (0.4)	0.469 (3.1)
LowR2	0.434 (2.7)	0.484 (7.4)
NegR1	0.050 (2.8)	0.075 (18)
NegR2	0.057 (14.8)	0.072 (6.8)

*Optical density (% deviation)

Table 24 shows that the values of optical density obtained by utilizing sensitized plates with the second lot of polyclonal antibody are between the upper and lower limits established for each of the lot of antigens in section III-1. Therefore, the purification of the polyclonal antibody was carried out and validated a second time. It may be concluded that the method of purification of the polyclonal antibody is reproducible.

9. Reproducibility of the production of MAb

The reproducibility of the method of production of MAb was tested.

Two lots of different MAb have been produced according to the method described in section I-2. The ELISA test was realized by utilizing as antigens the panel of antigen (section III-1) as well as C+ and C-. Each MAb was tested against all the antigens. The working dilution of each MAb was established as described in section II-2. The MAb lot #SE2192M89C was used as control of the reaction.

Table 25

Reproducibility of the production of MAb

Antigens	MAb		
	Lot #SE2192M89C	Lot #AL1194M89C	Lot #AL2694M89C
C +	1.650 (3.8)*	1.578 (2.4)	1.639 (1.2)
C -	0.058 (4.8)	0.060 (7)	0.067 (3.1)
HighR1	1.478 (2.1)	1.485 (1.3)	1.495 (0.7)
HighR2	1.393 (1.2)	1.404 (5.5)	1.466 (1.1)
LowR1	0.472 (4.9)	0.466 (1)	0.474 (1.3)
LowR2	0.370 (1.1)	0.370 (2.1)	0.379 (1.4)
NegR1	0.061 (5.8)	0.061 (3.5)	0.069 (5.1)
NegR2	0.049 (11.5)	0.051 (0)	0.060 (7)

*Optical density (% deviation)

Table 25 shows that the values of optical density obtained by utilizing the three lots of MAb are found between the upper and lower limits established for each of the lots of antigens in section III-1. Therefore, the production of MAb was realized and validated three times. It can be concluded that the method of production of the MAb is reproducible.

IV- STABILITY OF KITS, CONDITIONS OF PRESERVATION

The first objective was to establish adequate conditions of preservation for each element of the kit (points 1 to 7, below). For this purpose, each of the elements composing the kit was preserved at 4°C under different conditions. The lyophilized and non-lyophilized elements, as well as resuspended in different solutions of dilution being tested during 22 weeks. The tests were realized by utilizing all of the freshly prepared elements except the element to be tested. Moreover, the element to be tested, freshly prepared, was included in all the tests in order to be used as control of the reaction. The choice of the conditions of preservation was based on the following conditions:

- a minimum time of preservation of 22 weeks (based on the comparison of the values of optical density between the element to be tested and the reaction control),
- the conditions of non-lyophilization were preferred to the conditions of lyophilization; and
- facility of utilization in the field.

The second objective was to validate the kit made of the elements selected in points 1 to 7 (point 8, below).

The third objective was to establish the time of preservation of each element of the kit at 22 and 37°C (point 9, below).

The fourth objective was to establish the time of preservation of the elements of the kit after the kit has been opened (point 10, below).

1. The plates

The objective was to test the stability of the plates previously sensitized and blocked.

The plates were sensitized with the polyclonal antibody diluted 1/400 in PBS (1µg/well) during 1 h at 37°C. After 3 washings, the plates were blocked with TRIS-C-T during 30 min. at 37°C. The plates were closed with a sheet of acetate, sealed in an aluminum wrapper and preserved at 4°C.

The plates were tested at weeks 10 and 22 after their preparation. A sensitized plate and blocked the day of the test was used as control plate.

Table 26

Stability of sensitized plates at 4°C

N° of weeks	Antigen	Plates at 4°C	Control plates
0	C+	1.479 (2.25)*	0.870 (2.84)
	C-	0.107 (7.93)	0.081 (28.9)
10	C+	1.178 (0.48)	1.248 (3.57)
	C -	0.049 (2.89)	0.074 (13.3)
22	C +	1.452 (1.85)	1.383 (0.09)
	C -	0.029 (4.88)	0.071 (1.99)

*Optical density (% deviation)

Table 25 shows that the plates previously sensitized and blocked preserved at 4°C give values of optical density equivalent to those of the control plates. Therefore, the plates preserved at 4°C appear stable at least during 22 weeks.

2. Positive Control (virus BRSV)

The objective was to test the stability of positive control kept at 4°C.

The BRSV Lot #OE1292VRS was diluted at the rate of 2:1, 1:1 and 1:5 in SPGA (0.218M sucrose, 0.0038M $KHPO_4$, 0.0072M K_2PO_4, 0.0049M sodium glutamate, pH between 6.2 and 7). These viral preparations, lyophilized and non-lyophilized were kept at 4°C.

Moreover, BRSV was diluted at the rate of 1:1, 4:1 and 9:1 in the stabilizing solution (SS) (HRP conjugate #516534, Calbiochem, La Jolla, CA) and kept non-lyophilized at 4°C.

The positive controls that were tested at weeks 10 and 22 after their preparation. A positive control unfrozen the day after the test was utilized as reaction control.

Table 27

Stability of the positive control lyophilized and kept at 4°C

Weeks	BRSV dilution factor in SPGA solution				Control
	Pure	2:1	1:1	1:5	
0	2.036 (1.5)*	1.448 (0)	1.079 (1.7)	0.907 (6.5)	1.026 (2.76)
10	1.881 (6.9)	1.316 (0.7)	1.099 (1.4)	1.263 (0.2)	1.248 (3.5)
22	1.970 (1.2)	1.936 (0.8)	1.292 (1.7)	1.193 (12.6)	1.383 (0.1)

*Optical density (% deviation)

Table 27 shows that the positive controls lyophilized and kept at 4°C give values of optical density which are equivalent to those of the controls freshly prepared. Therefore, the positive controls which are lyophilized and kept at 4°C appear stable at least during 22 weeks.

All the four conditions tested may be selected to preserve the positive control of the kit. Pure

lyophilized BRSV were selected because it is not diluted and gives the highest optical density.

Table 28

Stability of positive control non-lyophilized at 4°C

Weeks	BRSV dilution factor in the SPGA solution			Control	BRSV dilution factor in the SS solution			Control
	2:1	1:1	1:5		1:1	4:1	9:1	
0	1.175 (4.6)*	1.130 (1.4)	0.958 (4.4)	1.026 (2.7)	1.699 (1.0)	1.758 (4.9)	1.706 (3.5)	1.345 (22.4)
7.5					1.180 (1.5)	1.617 (5.9)	1.247	1.407 (2.6)
10	0.396 (4.2)	0.206 (0.3)	0.102 (7.6)	1.248 (3.5)				

*Optical density (% deviation)

Table 28 shows that the positive controls, non-lyophilized and diluted in the SPGA solution are not stable at 4°C. However, when the positive control is diluted in the SS solution, it appears stable at least during 10 weeks. A follow up of these positive controls during 22 weeks offers the future possibility of utilizing a non-lyophilized positive control.

3. Negative Control

The objective was to verify the stability of the negative control kept at 4°C.

The cells MDBK Lot #AL1192CMDBK were diluted at the rate of 2:1, 1:1 and 1:5 in SPGA. These preparations were kept at 4°C, lyophilized and non-lyophilized.

The negative controls were verified at weeks 10 and 22 after their preparation. Cells which were unfrozen the day of the test were used as control for the reaction.

Table 29

Stability of the negative controlled lyophilized and kept at 4°C

Weeks	Dilution factor of the negative control in the SPGA solution				Control
	Pure	2:1	1:1	1:5	
0	0.074 (20.2)*	0.069 (16.4)	0.067 (20.2)	0.068 (23.7)	0.078 (5.4)
10	0.078 (13.6)	0.068 (13.6)	0.065 (21.7)	0.059 (2.4)	0.074 (13.3)
22	0.074 (9.5)	0.071 (0)	0.073 (4.8)	0.074 (9.5)	0.071 (1.9)

*Optical density (% deviation)

Table 30

Stability of non-lyophilized negative control at 4°C

Weeks	Dilution factor of the negative control in the SPGA solution			Control
	2:1	1:1	1:5	
0	0.081 (19.2)*	0.074 (5.7)	0.069 (11.3)	0.078 (5.4)
10	0.093 (25.8)	0.102 (16.0)	0.097 (34.9)	0.074 (13.3)

*Optical density (% deviation)

Tables 29 and 30 show that the negative controls kept under all the conditions tested gave the values of optical density which are equivalent to those of the controls freshly prepared. In order to keep the same conditions as in the positive control, the lyophilized pure cells were selected as negative control of the kit.

4. Monoclonal Antibody (MAb)

The stability of the MAb kept at 4°C was to verified.

The MAb Lot SE2192M89C was diluted 1/4 and 1/8 in the SPGA solution and in TRIS 50mM pH 8.6. These preparations were kept at 4°C lyophilized and non-lyophilized.

The MAb was verified at weeks 10 and 22. The MAb which was unfrozen the day of the test were used as control of the reaction.

Table 31

Stability of the MAb at 4°C

Weeks	Anti-gens	Dilution factor of the lyophilized MAb				Dilution factor of the non-lyophilized MAb				Control
		1/8 TRIS	1/4 TRIS	1/8 SPGA	1/4 SPGA	1/8 TRIS	1/4 TRIS	1/8 SPGA	1/4 SPGA	
0	C+	1.079 (6.0)*	1.130 (3.4)	1.122 (0.3)	1.151 (4.4)	1.120 (4.3)	1.116 (2.5)	1.091 (4.8)	1.026 (8.5)	1.003 (10.1)
	C-	0.095 (6.7)	0.087 (6.5)	0.114 (2.4)	0.111 (1.2)	0.095 (2.2)	0.085 (3.3)	0.123 (4.0)	0.098 (2.8)	0.101 (11.2)
10	C+	1.123 (0)	1.152 (0.3)	1.082 (1)	1.144 (0.6)	1.145 (2.2)	1.662 (0.8)	1.082 (0)	1.127 (0.7)	1.174 (2.7)
	C-	0.059 (10.8)	0.062 (15.9)	0.072 (14.8)	0.083 (9.4)	0.063 (14.7)	0.066 (14)	0.065 (2.1)	0.073 (9.6)	0.064 (5.5)
22	C+	1.394 (0.7)	1.431 (1.9)	1.483 (1.2)	1.352 (1.1)	1.444 (1.9)	1.517 (2)	1.388 (4.9)	1.276 (4.7)	1.383 (0)
	C-	0.073 (10.7)	0.077 (8.3)	0.093 (0.7)	0.096 (0.7)	0.066 (0)	0.076 (4.6)	0.087 (3.2)	0.085 (4.9)	0.07 (1.9)

*Optical density (% deviation)

Table 31 shows that the lyophilized and non-lyophilized MAb gives values of optical density which are equivalent to those of the freshly prepared MAb. Therefore, the MAb appears stable at least during 22 weeks.

All the conditions tested may be selected to preserve the MAb of the kit. MAb non-lyophilized and diluted 1/4 in TRIS was selected .

5. The Conjugate

The stability of the conjugate kept at 4°C was to verified.

The conjugate Lot #115-035-062 was diluted at the rate 1/100 and 1/500 in TRIS-C-T in the presence of

1% casein or 3% albumin or 1% gelatin or 10% bovine fetal serum (SFB) or 1% calf serum.

The conjugate was also diluted 1/100 in the SS solution.

These preparations were kept at 4°C lyophilized and non-lyophilized.

The conjugate was verified at weeks 10 and 22. The conjugate unfrozen the day of the test was used as control of the reaction. The conjugates 1/100 were diluted at 1/3000 before their use.

Table 32

Stability of the conjugate lyophilized at 4°C

Weeks	Anti-gen	Dilution factor of the conjugate in different solutions								Control
		1/100 TCT	1/500 TCT	1/3000 TCT	1/100 1% of casein	1/100 3% of albumin	1/100 1% of gelatin	1/100 10% of SFB	1/100 1% calf serum	
0	C+	0.994 (2.3)*	0.957 (5.7)	0.633 (4)	0.818 (1.5)	1.194 (2.6)	1.153 (6)	1.344 (1.7)	0.953 (0.3)	1.204 (0.2)
	C-	0.128 (4.4)	0.111 (12.7)	0.054 (18.3)	0.092 (10)	0.124 (1.7)	0.136 (6.2)	0.144 (1.9)	0.130 (6.5)	0.126 (3.3)
10	C+	1.181 (3.5)	0.866 (2.5)		1.098 (0.5)	0.892 (0.1)	1.443 (3)	1.420 (4.9)	1.152 (1.7)	1.515 (6.3)
	C-	0.09 (12.5)	0.052 (10.8)		0.077 (18.3)	0.052 (0)	0.105 (6)	0.111 (28.8)	0.101 (4.8)	0.071 (3)
22	C+	1.023 (3.2)			1.704 (0.2)	1.358 (1.3)	1.738 (2.7)	0.284 (3.4)	1.206 (0.4)	1.457 (2.6)
	C-	0.06 (12.8)			0.136 (4.1)	0.092 (3)	0.142 (0)	0.010 (6.7)	0.103 (3.4)	0.088 (0)

*Optical density (% deviation)

Table 32 shows that the conjugate diluted in TRIS-CT contains either 1% casein or 3% albumin or 1% gelatin, and when lyophilized gives values of optical density which are equivalent to those the freshly prepared conjugate. Therefore, the conjugate under the above-described conditions, appear stable at least during 22 weeks.

Table 33

Stability of the non-lyophilized conjugate at 4°C

Weeks	Anti-gens	Dilution factor of the conjugate in different solutions							Control
		1/100 TCT	1/100 1% of casein	1/100 3% of albumin	1/100 1% of gelatin	1/100 10% of SFB	1/100 1% calf serum	1/100 SS	
0	C+	0.928 (5.3)*	0.884 (8.8)	0.957 (3.1)	0.920 (8.6)	0.727 (3)	0.843 (1.7)	1.181 (14)	1.314 (1.4)
	C-	0.073 (1.9)	0.081 (11.2)	0.078 (4.5)	0.085 (19.9)	0.076 (0.9)	0.073 (12.6)	0.164 (2.3)	0.126 (2.8)
10	C+	0.167 (0.4)	0.135 (3.1)	0.213 (2.9)	0.263 (1.3)	0.228 (2.8)	0.197 (1.4)		1.455 (2.3)
	C-	0.004 (35)	0.007 (0)	0.008 (9.4)	0.010 (7.4)	0.004 (35)	0.008 (9.4)		0.068 (7.2)
22	C+							1.212 (4.2)	1.241 (0)
	C-							0.067 (1)	0.064 (8)

*Optical density (% deviation)

Table 33 shows that the conjugate, non-lyophilized and kept at 4°C, gives values of optical density which are equivalent to those of the controlled conjugate only when it is diluted solution SS. Therefore, the conjugate, non-lyophilized and diluted 1/100 in the SS solution and kept at 4°C, has been chosen as the conjugate of the kit.

6. The Solutions

The stability of the washing solution, the solution of dilution MAb, TRIS-C-T, chromogenic substrate kept at 4°C was verified.

The solutions designated above were kept at 4°C. The solutions were verified at months 3 and 12 after their preparation. The freshly prepared solutions were used as reaction control.

Table 34

Stability of solutions kept at 4°C

N° of months	Antigen	Solutions at 4°C	Control solutions
3	C +[1]	0.976	0.970
	C -	0.076	0.100
12	C+	1.245	1.255
	C -	0.078	0.041

*Optical density (Deviation %)

[1]BRSV Lot #OE1292VRS diluted 1/10 in the TRIS pH 8.4

Table 34 shows that the solutions kept at 4°C give values of optical density which are equivalent to those of freshly prepared solutions. Therefore, the solutions appear stable at least during 12 months at 4°C.

7. Conclusion:
Selection of the method of preservation for each element of the kit

The objective was to select the method of preservation of each component of the kit.

The following choice was made in accordance with the results presented in the preceding points:

-Positive control: BRSV pure lyophilized at 4°C.
-Negative control: cells MDBK pure lyophilized at 4°C.
-MAb: diluted 1/4 in TRIS non-lyophilized at 4°C.
-Conjugate: diluted 1/100 in the stabilizing solution, non-lyophilized at 4°C.

All these elements were combined to constitute kits. The kit thus assembled was validated at 5 months and 7.5 months after its preparation (see point 8, below).

8. Validation of 5- and 7.5-month kits

The objective was to validate kits composed of elements preserved during 5 and 7.5 months at 4°C.

The ELISA test was carried out by utilizing as antigens the panel of antigens (section III-1) as well as C+, C-, a positive sample and a negative sample. The values of the optical density obtained with the panel of antigen was compared to those fixed as low limit and upper limit in section III-1. The values of optical density obtained with C+, C-, the positive sample and negative sample were compared to those obtained by utilizing a freshly prepared kit.

Table 35

Validation of kits of 5- and 7.5-month

Antigen	5-month kit	Control kit* (5 month)	7,5-month kit	Control kit (7.5-month)	Lower DO limit	Upper DO limit
C +	1.547	1.698	1.179	1.304	0.829	1.885
C -	0.101	0.107	0.019	0.055	0.003	0.147
PC	1.608					
NC	0.099					
Sample +	0.460	0.396				
Sample -	0.063	0.042				
HighR1	1.545		1.442	1.538	1.301	1.955
HighR2	1.615		1.565	1.683	1.372	2.188
LowR1	0.485		0.396	0.428	0.380	0.686
LowR2	0.421		0.564	0.642	0.324	0.570
NegR1	0.080		0.028	0.055	0.032	0.122
NegR2	0.086		0.020	0.059	0.006	0.156

*Kit prepared on the day of testing
**Lower and upper DO limits fixed for the antigen panel in objective 3, point 1
PC: Control + of the 5 et 7.5 month kits;
NC: Control - of the 5 et 7.5 month kits.

Table 35 shows that the values of optical density by utilizing the kits kept during 5 and 7.5 months at 4°C are found between the superior and lower limits established for each of antigens composing the panel of antigens in section III-1. Moreover, the values of optical density obtained are equivalent to those obtained with the control kit, given a difference of

optical density <0.1. Therefore, it can be concluded that 5 and 7.5 month old kits are validated.

9. Stability of the elements composing the kit at 37 and 22°C

The objective was to test at different temperatures the stability of the selected kit.

Kits have been prepared and kept at 37°C, 22°C and 4°C. Each element of the kit was tested on days 1.5, 3, 9, 14, 20 and 32 after their preparation. The tests were realized by utilizing all the freshly prepared elements except for the element to be tested. The values of optical density obtained with the elements at 37°C and at 22°C were compared to the values obtained with the elements at 4°C. The values have been considered as equivalent if the difference was ≤ 0.1.

Table 36

Stability of the plates at 37 and 22°C

Number of days	Antigen	Plates at 37°C	Plates at 22°C	Plate at 4°C
0	C +	1.455	1.455	1.455
	C -	0.085	0.085	0.085
1.5	C +	1.663	1.547	1.549
	C -	0.049	0.066	0.077
3	C +	1.345	1.577	1.567
	C -	0.041	0.060	0.069
9	C +	0.932	1.436	1.490
	C -	0.023	0.044	0.054
14	C +	0.945	1.427	1.529
	C -	0.025	0.043	0.062
20	C +		1.308	1.388
	C -		0.051	0.056
32	C +		1.224	1.441
	C -		0.031	0.053

Table 36 shows that the plates are stable during 1.5 days at 37°C and during at least 20 days at 22°C.

Table 37

Stability of the positive and negative controls at 37 and 22°C

Number of days	Antigen	Antigen at 37°C	Antigen at 22°C	Antigens at 4°C
0	C +	1.675	1.675	1.455
	C -	0.069	0.069	0.085
1.5	C +	0.694	1.771	1.549
	C -	0.071	0.073	0.077
3	C +	0.624	1.719	1.567
	C -	0.077	0.073	0.069
9	C +	0.428	0.724	1.490
	C -	0.069	0.065	0.054
14	C +	0.215	0.432	1.529
	C -	0.035	0.038	0.062
20	C +	0.125	0.479	1.686
	C -	0.021	0.036	0.048
32	C +	0.202	0.512	1.623
	C -	0.048	0.052	0.052

Table 37 shows that the positive control has a decrease of 58% of its intensity of reaction after 1.5 days at 37°C and that it appears to be stable during 3 days at 22°C. However, even if after 32 days at 22°C the optical density represents 31% of the optical density of the control, it may be used as positive control of the kit since it gives a positive reaction upon visual reading. The negative control appears stable at least during 32 days at 22°C.

Table 38

Stability of MAb at 37 and 22°C

Number of days	Antigen	MAb at 37°C	MAb at 22°C	MAb at 4°C
0	C +	1.450	1.450	1.455
	C -	0.089	0.089	0.085
1.5	C +	1.541	1.619	1.678
	C -	0.068	0.071	0.189
3	C +	1.679	1.690	1.691
	C -	0.085	0.078	0.083
9	C +	1.609	1.694	1.765
	C -	0.068	0.073	0.078
14	C +	1.331	1.342	1.413
	C -	0.037	0.042	0.042
20	C +	0.876	1.514	1.621
	C -	0.023	0.040	0.050
32	C +		1.493	1.508
	C -		0.048	0.061

Table 38 shows that MAb is stable 14 days at 37°C and at least during 32 days at 22°C.

Table 39

Stability of the conjugate at 37 and 22°C

Number of days	Antigen	Conjugate at 37°C	Conjugate at 22°C	Conjugate at 4°C
0	C +	1.440	1.440	1.304
	C -	0.070	0.070	0.055
1.5	C +	1.324	1.354	1.400
	C -	0.060	0.063	0.058
3	C +	1.250	1.416	1.367
	C -	0.097	0.107	0.101
9	C +	1.134	1.357	1.432
	C -	0.065	0.095	0.094
14	C +	1.207	1.341	1.401
	C -	0.034	0.062	0.064
20	C +	1.069	1.284	1.356
	C -	0.073	0.088	0.092
32	C +	0.873	1.161	1.456
	C -	0.040	0.076	0.103

Table 39 shows that the conjugate is stable during 1.5 days at 37°C and during 20 days at 22°C.

Table 40

Stability of chromogenic substrate at 37 and 22°C

Number of days	Antigen	Chrom. Sub. at 37°C	Chrom. Sub. at 22°C	Chrom. Sub. at 4°C
0	C +	1.450	1.450	1.455
	C -	0.085	0.085	0.085
1.5	C +	1.421	1.437	1.454
	C -	0.063	0.064	0.066
3	C +	1.617	1.572	1.626
	C -	0.072	0.073	0.068
9	C +	1.392	1.525	1.495
	C -	0.064	0.065	0.070
14	C +	1.130	1.223	1.243
	C -	0.035	0.039	0.040
20	C +	0.795	1.475	1.567
	C -	0.020	0.044	0.053
32	C +		1.385	1.374
	C -		0.053	0.050

Table 40 shows that the chromogenic substrate are stable during 9 days at 37°C and during at least 32 days at 22°C.

Table 41

Stability of the washing solution at 37 and 22°C

Number of days	Antigen	Washing Solution at 37°C	Washing Solution at 22°C	Washing Solution at 4°C
20	C +	1.297	1.360	1.398
	C -	0.050	0.044	0.042

Table 41 shows that the washing solution is stable during at least 20 days at 37 and 22°C.

10. Stability of the elements making up the kit after its opening.

The objective was to test the stability of each element of the kit after its opening, i.e. after a first utilization.

A kit was opened at kept at 4°C. Each element of the kit was tested on days 1.5, 3, 9, 14, 20 and 32 after opening of the kit. The tests were carried out by utilizing all the freshly prepared elements except for the element to be tested. The values of optical density obtained were compared with the values obtained on days 0. The values were considered as equivalent if the different was ≤ 0.1.

Table 42

Stability of the elements of the kit after its opening

No. of days	Antigen	Plate	Controls + and -	MAb	Conjugate	Sub-Chrom
0	C +	1.455	1.608	1.455	1.304	1.455
	C -	0.085	0.099	0.085	0.055	0.085
1.5	C +	1.549		1.678	1.400	1.454
	C -	0.077		0.189	0.058	0.066
3	C +	1.567	1.745	1.691	1.367	1.626
	C -	0.069	0.062	0.083	0.101	0.068
9	C +	1.490	1.478	1.765	1.432	1.495
	C -	0.054	0.060	0.078	0.094	0.070
14	C +	1.529		1.413	1.401	1.243
	C -	0.062		0.042	0.064	0.040
20	C +	1.388		1.621	1.356	1.567
	C -	0.056		0.050	0.092	0.053
32	C +	1.441	0.920*	1.508	1.456	1.374
	C -	0.053	0.056	0.061	0.103	0.050

*The quantity being insufficient, the test was carried out with 30µl of control instead of 100µl

Table 42 shows that the values of optical density between day 0 and day 32 are equivalent since the difference found was ≤ 0.1. Therefore, it can be con-

cluded that the elements of the kit are stable at least during 32 days after the opening of the kit.

CONCLUSION

The production and the purification of bovine polyclonal antibodies which are specific to BRSV have been carried out.

The production of MAb which are specific to BRSV was carried out and MAb RSQ89C was selected as a second antibody for double sandwich ELISA.

The setting up of the ELISA technique for the detection of BRSV was carried out.

An evaluation of the ELISA test developed shows:

- the results are not influenced by the arrangement on the plate (study of the variation intra-plate).
- the results are not influenced by the utilization of different plates (study of the variation inter-plate).
- the immunological sensitivity of ELISA was evaluated at 10^2 $DICT_{50}$.
- when 18 different viral strains were tested, the ELISA was specific to RSV.
- the relative sensitivity and specificity were 92% and 100% respectively, when the viral isolation on cellular culture was used as reference test.

The conditions of presentation and preservation of the ELISA kits as well as their period of preservation were established.

SAMPLING OF NASAL SECRETIONS

Collecting the sample by means of a new sponge about 2.5 cm X 2.5 cm X 2.5 cm.

Deeply insert the sponge in one nostril of the animal. Ensure that it is well in place and that the cord is outside to enable its removal. Leaving the sponge on the animal during 15 minutes.

Removing the sponge, removing the piston of the syringe and placing the sponge. Slowly replacing the piston. Preventing any pressing of the sponge.

Allowing the sponge in the syringe until the test is carried out. It is preferably that the sample and the test be carried out the same day, otherwise, freezing at -70°C.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

WE CLAIM:

1. An ELISA test for qualitatively determining the presence of living or inactivated BRSV antigen in a bovine biological sample, wherein said sample has an unknown amount of BRSV antigen, which comprises the following:

a) incubating a solid support having bound thereto a first anti-BRSV antibody with said biological sample for a time sufficient for an immune complex to form between the anti-BRSV antibody and any BRSV antigen present in said sample;

b) incubating said incubated solid support of step a) with a second anti-BRSV antibody; and

c) detecting the bound second antibody of step b) to determine the quantity of the BRSV antigen present in said sample.

2. The ELISA of claim 1, which further comprises a step consisting of washing between steps a) and b).

3. The ELISA of claim 2, which further comprises a step before step b), wherein said step consists of reacting said washed solid support of step a) with a blocking solution to obstruct sites the non-covered by the first antibody, thereby preventing adhesion of any other particle on the solid support.

4. The ELISA of claim 3, wherein said blocking solution is selected from the group consisting of TRIS-casein-TWEEN™ and PBS-TWEEN™-thimerosal.

5. The ELISA of claim 4, which further comprises a step consisting of washing before step b).

6. The ELISA of claim 5, which further comprises a step consisting of washing between steps b) and c).

7. The ELISA of claim 6, wherein said first antibody is a polyclonal antibody.

8. The ELISA of claim 7, wherein said second antibody is a monoclonal antibody.

9. The ELISA of claim 8, wherein detecting carried out in step c) is effected by fixing an anti-mouse antibody conjugated to peroxidase on the bound antibody.

10. The ELISA of claim 9, which further comprises a step consisting of washing after fixing said conjugated peroxidase.

11. The ELISA of claim 10, wherein said peroxidase develop a blue coloration upon addition of the chromogenic substrates.

12. The ELISA of claim 11, wherein said bovine biological sample consists in nasal secretions.

13. The ELISA of claim 12, wherein the intensity of said bleu color is read visually or by a spectrophotometer after addition of a stopping solution.

14. The ELISA of claim 13, wherein the stopping solution is H_2SO_4.

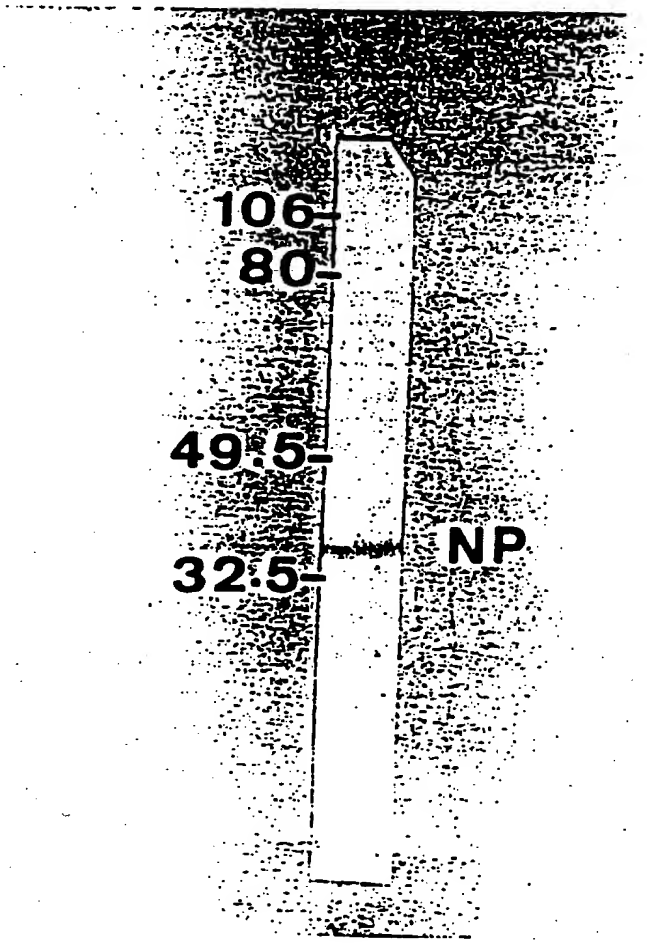
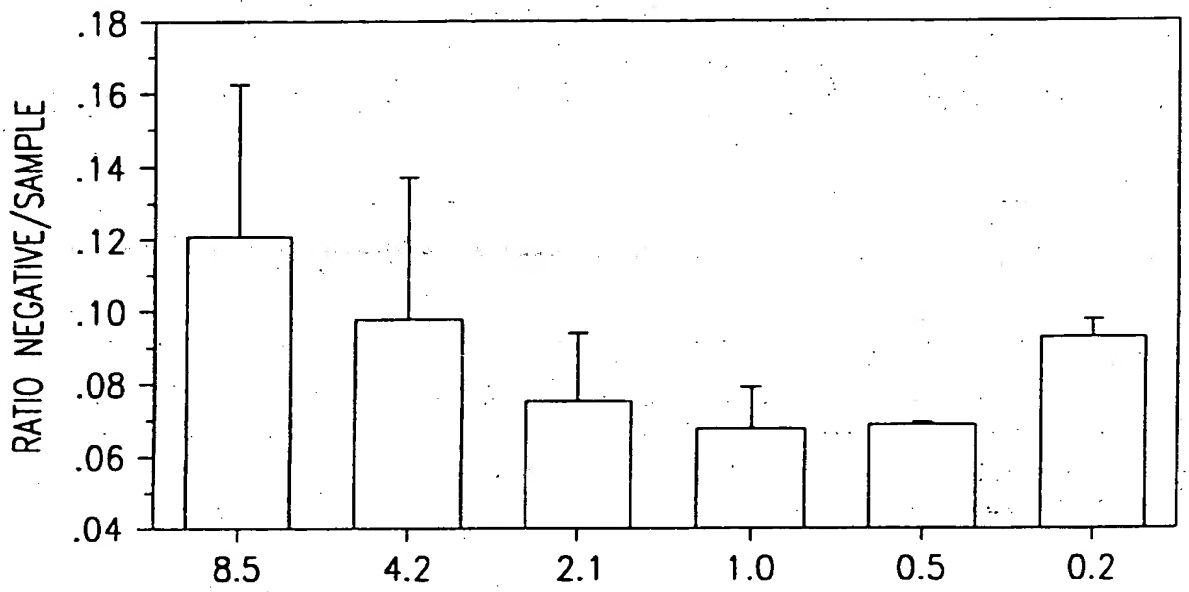


FIG. 1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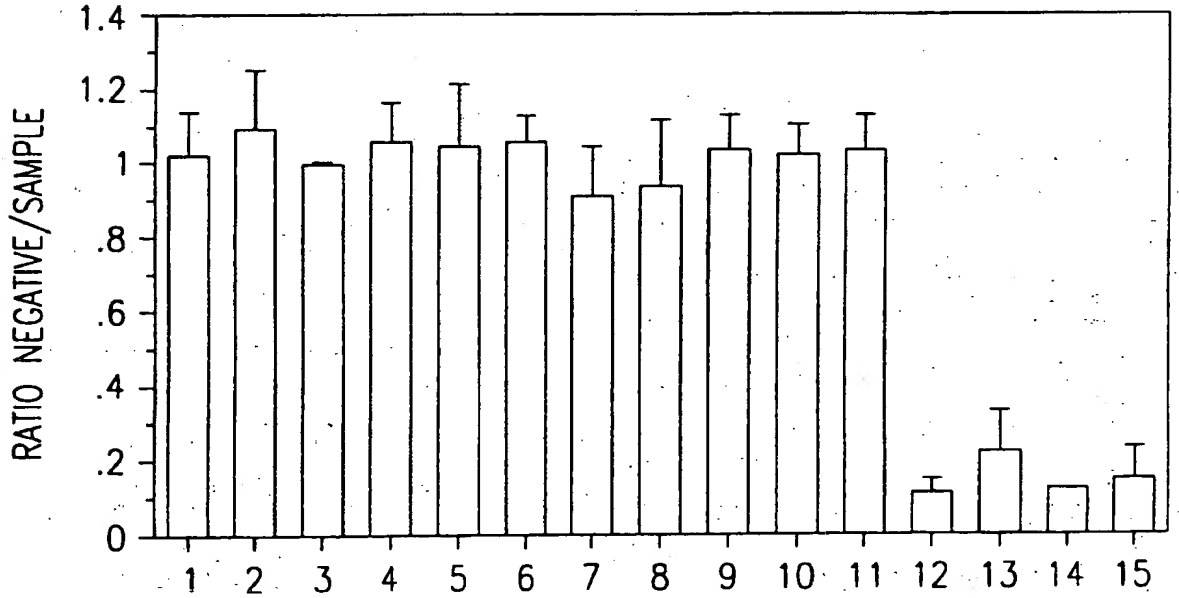
0.5
0.2

FIG_2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.2
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1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

FIG_3

INTERNATIONAL SEARCH REPORT

International Application No
PCT/U.. 96/00662

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/569 G01N33/58

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOURNAL OF IMMUNOLOGICAL METHODS, vol. 77, 1985, pages 247-258, XP002024899 R. M. HENDRY ET AL.: "Monoclonal capture antibody ELISA for Respiratory Syncytial Virus: detection of individual viral antigens and determination of monoclonal antibody specificities. " see the whole document --- -/--	1-14

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 February 1997	27 -02- 1997

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Cartagena y Abella,P

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOURNAL OF VIROLOGICAL METHODS, vol. 17, no. 3,4, September 1987, pages 247-261, XP000617816 A. CLAYTON ET AL.: "The selection and performance of monoclonal and polyclonal anti-respiratory syncytial virus (RS) antibodies in capture ELISAs for antigen detection." see the whole document	1-14
A	EP,A,0 242 082 (CITY OF LONDON POLYTECHNIC) 21 October 1987 see claims 1,4,5	4
A	DATABASE WPI Week 8809 Derwent Publications Ltd., London, GB; AN 88-060559 XP002024900 & JP,A,63 016 266 (TOYOBO KK) , 23 January 1988 see abstract	4
A	DATABASE WPI Section Ch, Week 9429 Derwent Publications Ltd., London, GB; Class B04, AN 94-240423 XP002024901 & SU,A,1 814 075 (MOTHER CHILD PROTECTION RES. INST.) , 7 May 1993 see abstract	14

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/C.. 96/00662

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-242082	21-10-87	GB-A- 2189317	21-10-87
		JP-A- 62289766	16-12-87